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Prenylated Stilbenes from Peanut Root Mucilage†

VICTOR S. SOBOLEV,[1]* THOMAS L. POTTER[2] and BRUCE W. HORN[1]

[1] National Peanut Research Laboratory, Agricultural Research Service, United States Department of Agriculture, PO Box 509, Dawson, Georgia 39842, USA

[2] Southeast Watershed Research Laboratory, Agricultural Research Service, United States Department of Agriculture, PO Box 748, Tifton, GA 31793, USA

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Abstract: Seven prenylated stilbenes were identified by combined HPLC-PAD-APCI/MS^n analysis of an extract of mucilage isolated from peanut (*Arachis hypogaea* L.) root tips. The principal constituent was assigned the structure 4-(3-methyl-but-1-enyl)-3, 5-dimethoxy-4′-hydroxy-*trans*-stilbene. The common name mucilagin A is proposed for this novel compound. Its concentration in the mucilage was estimated at 250 μg/g (wet weight basis). The large body of literature on the anti-microbial properties of plant-derived stilbenes suggests that compounds detected in peanut mucilage may play a role in regulating root–soil pathogen interactions. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: HPLC-MS; stilbenes; mucilage; peanuts; groundnuts; *Arachis hypogaea*.

INTRODUCTION

A mucilaginous external layer (mucilage) is often observed on plant root tips. Mucilage consists of sloughed organic matter produced by roots, and is reported to promote formation of mycorrhizae and nitrogen-fixing nodules, to enhance nutrient and water absorption, promote soil aggregation, reduce root desiccation during drought and mechanically to lubricate the roots (Greaves and Darbyshire, 1972; Bowen and Rovira, 1976; Griffin *et al.*, 1976; Paull and Jones, 1976; Werker and Kislev, 1978; Foster, 1982; Rougier and Chaboud, 1985; McCully, 1999; Walker *et al.*, 2003). Excretion of anti-microbial compounds by roots, particularly in response to elicitors such as pathogenic fungi, has been documented in many species (Flores *et al.*, 1999; Walker *et al.*, 2003; Bais *et al.*, 2004). For example, Bais (2002) identified rosmarinic acid in exudates of hairy root cultures of sweet basil (*Ocimum basilicum*) when treated with cell-wall extracts of *Phytophthora cinnamoni* or challenged *in situ* with *Pythium ultimum*. They also demonstrated that rosmarinic acid has potent activity against many soil fungi and bacteria.

Several potent anti-microbials, including resveratrol, isopentenyl and isopentadienyl resveratrol analogues, have been isolated from peanut leaves, stems, pegs, roots and kernels when challenged by pathogens (Ingham, 1976; Keen and Ingham, 1976; Aguamah *et al.*, 1981; Strange *et al.*, 1985; Cooksey *et al.*, 1988; Dorner *et al.*, 1989; Arora and Strange, 1991; Edwards and Strange, 1991; Sobolev *et al.*, 1995). Relatively high levels of resveratrol have been reported in dried roots of some peanut varieties (Chen *et al.*, 2002). It is likely that these compounds play a defensive role when the water activity level in plant tissues is sufficiently high (Dorner *et al.*, 1989). Indirect evidence for this defensive role is that aflatoxin contamination, resulting from *Aspergillus* spp. infection, increases as peanut kernels lose their capacity for phytoalexin synthesis as a result of dehydration under drought conditions (Dorner *et al.*, 1989).

Peanut mucilage may play a role in the interactions between roots and soil-borne plant pathogens (Rougier and Chaboud, 1985); however, the occurrence of phytoalexins or preformed anti-microbial compounds in peanut mucilage has not been reported.

Mass-spectrometry has frequently been used as a characterisation tool for the analysis of stilbene derivatives (Budzikiewicz *et al.*, 1967; Keen and Ingham, 1976; Edwards and Strange, 1991; Schultz *et al.*, 1992; Ioset *et al.*, 2001; Su *et al.*, 2002). Various stilbenes with known structures may be isolated from natural sources (Keen and Ingham, 1976; Aguamah *et al.*, 1981; Cooksey *et al.*, 1988; Sobolev *et al.*, 1995) and serve as reference compounds for MS^n structural characterisation of new stilbenes. Such an approach has been used in the present study in which novel prenylated stilbenes isolated from peanut mucilage are described.

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* Correspondence to: V. S. Sobolev, National Peanut Research Laboratory, Agricultural Research Service, United States Department of Agriculture, PO Box 509, Dawson, Georgia 39842, USA.
E-mail: vsobolev@nprl.usda.gov

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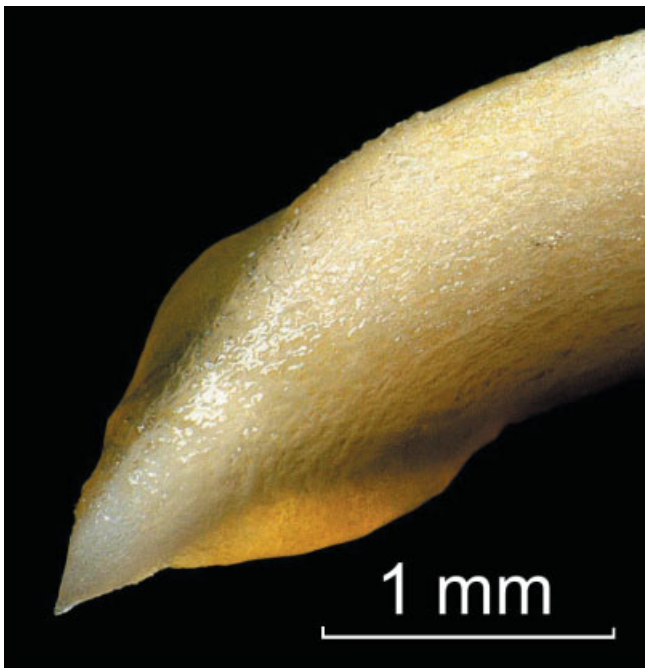

Figure 1 Photomicrograph of peanut root tip showing the yellow mucilage layer. This figure is available in colour online at www.interscience.wiley.com/journal/pca

EXPERIMENTAL

Reagents. Celite and the HPLC-grade solvents (methanol, toluene, acetone, water and formic acid) used in the preparation of mobile phases and in sample extraction and methylation were obtained from Fisher (Suwanee, GA, USA). Dimethyl sulphate (>99%), ammonium hydroxide (28%), anhydrous potassium carbonate and anhydrous sodium sulphate were purchased from Aldrich (Milwaukee, WI, USA). Media for peanut germination was composed of 1% agar (Difco Laboratories, Sparks, MD, USA) in sterile water.

Plant material and processing. Peanuts (*Arachis hypogaea* L; cultivar Georgia Green) were harvested in 2001 in Shellman, GA, USA. Dried pods were sterilised by gentle shaking with 2% sodium hypochlorite for 2 min followed by drying at room temperature in covered sterile trays. Kernels were aseptically removed from the pods and those without injury or discoloration were germinated on 1% water agar in 100 × 15 mm plates (three seeds/plate) under axenic conditions for 5 days at 30°C in darkness. Mucilage formed as a layer (Fig. 1) on peanut root tips and contained sloughed cells, as described by Griffin *et al.* (1976). The layer could be easily and quickly removed as a single mass from the root tip with a small spatula. Mucilage from root tips of 107 germinated kernels was removed (0.08–0.31 g total fresh weight), combined and stored at –20°C. In order to ensure that freezing did not affect the quality and quantity of the extracted secondary metabolites, mucilage from a separate set of 10 root tips (three replicates) was removed and immediately ground with celite as described below, and the extracting solvent added; the entire procedure was completed within 2 min in order to minimise possible enzymatic reactions. The results were similar to those obtained for the frozen samples.

Extraction cleanup. Mucilage (0.3 g) was thawed, ground with an equal amount of celite in an agate mortar and then extracted with 1.5 mL acetonitrile:water (90:10, v/v) for 5 min. The extract was filtered through a glass-fibre filter and 0.5 mL of the filtrate applied to a basic alumina (200 mg) micro-column, which was then washed with 0.3 mL of acetonitrile:water (90:10, v/v). The combined eluates were evaporated to dryness under a stream of nitrogen gas and the residue dissolved in 0.3 mL methanol for HPLC analysis.

HPLC-PAD analyses. Analyses were performed using a high-performance liquid chromatograph equipped with a pump (model LC-10ATvp; Shimadzu, Tokyo, Japan), a photodiode array detector (PAD; Shimadzu model SPD-M10Avp with Shimadzu Client/Server software, version 7.1.1) operating in the 200–600 nm range, and an autosampler (model 717 plus; Waters, Milford, MA, USA). The separation was performed on a Waters Spherisorb S3W analytical column (150 × 4.6 mm i.d.; packed with 3 µm silica gel) with *n*-hexane:2-propanol:water:acetic acid (2100:550:40:1, by volume) as the mobile phase at a flow rate of 1.2 mL/min at room temperature. A 'saturation' custom-made column (250 × 4.6 mm i.d.; packed with 63–200 µm silica gel; Universal Scientific, Atlanta, GA, USA) was installed in the solvent line between the pump and the autosampler in order to reduce silica gel solubility in the analytical column. The column was equilibrated with the mobile phase for 2 h at 1.5 mL/min.

HPLC-PAD-MS^n analyses. Analyses were performed using a SpectaSystem P-4000 HPLC pump linked in series to a SpectraSystem UV6000LC PAD (both from Thermoquest-Finnigan, San Jose, CA, USA) and an LCQ DECA ion trap mass spectrometer equipped with an atmospheric pressure chemical ionisation (APCI) interface and operated with Xcalibur™ version 1.2 software (Thermoquest-Finnegan). The HPLC was fitted with a Beckman (Alltech, Deerfield, IL, USA) Ultrasphere ODS column (150 × 4.6 mm; 5 µm) maintained at 40°C in a heated compartment within the autosampler (model AS-3000) (Thermoquest-Finnigan, San Jose, CA, USA) of the HPLC-MS system. Methanol (A) and 0.1% formic acid (B), adjusted to pH 3.5 with ammonium hydroxide, were combined in the following gradient: initial conditions 10:90 (A:B) held for 1 min, increased linearly to 90:10 in 15 min, held for 9 min and then decreased to initial conditions in 1 min. The

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mass flow rate was 1 mL/min. The PAD was scanned from 200 to 400 nm and the MS in the full-scan mode from m/z^+ 100 to 500. Prior to sample analysis, the MS response was optimised using a solution of *trans*-resveratrol (**1**) in methanol (10 mg/mL), which was infused into the HPLC column effluent at the rate of 15 μL/min. During tuning, the mobile phase composition was fixed at 50:50 (A:B) and the flow rate was 1 mL/min. The most intense response for the protonated parent ion $[M+H]^+$ (m/z^+ 229) was obtained with a vaporiser temperature of 450°C, a heated capillary temperature of 160°C, a discharge current of 6 μA, and a sheath gas flow of 40 units. These conditions were established prior to a software controlled autotune on this ion. In tandem MS (MS^2) analyses, the $[M+H]^+$ ion(s) observed for each chromatographic peak in the full-scan analyses were isolated and subjected to source collision induced dissociation (CID) using helium buffer gas. MS^3 analyses involved sequential isolation of $[M+H]^+$ ions and their most abundant CID fragment(s) identified in MS^2 analyses and CID. In all CID analyses, the isolation width, relative fragmentation energy, relative activation Q (RF frequency used for fragmentation) and activation time were, respectively, m/z^+ 1, 35%, 0.25 and 30 ms. CID product ions were detected by scanning the mass filter from 70 to 10 mass units above the m/z^+ of the isolated ion.

Reference compounds. Resveratrol (*t*-3,5,4′-trihydroxystilbene; approximately 99%) was purchased from Sigma (St. Louis, MO, USA). A reference mixture of peanut stilbenes containing resveratrol (**1**), *t*-4-(3-methyl-but-1-enyl)-3,5,3′,4′-tetrahydroxystilbene (*t*-arachidin-1; **3**), *t*-4-(3-methyl-but-2-enyl)-3,5,4′-trihydroxystilbene (*t*-arachidin-2; **4**), *t*-4-(3-methyl-but-1-enyl)-3,5,4′-trihydroxystilbene (*t*-arachidin-3; **5**) and *t*-3′-isopentadienyl-3,5,4′-trihydroxystilbene (*t*-IPD; **6**) was extracted from *Aspergillus flavus*-challenged peanut kernels using methods described by Sobolev *et al.* (1995).

Methylation. The mucilage extract and *t*-arachidin-3 (**5**), separated by TLC from the phytoalexin mixture derived from the *A. flavus* challenged peanut kernels, were methylated with dimethyl sulphate (DMS). This was accomplished by dissolving ca. 0.2 mg of sample in 0.6 mL acetone and combining the solution with 150 mg potassium carbonate and 50 μL of DMS in a 4 mL clear glass vial sealed with a Teflon-lined cap. The reaction mixture was held for 1 h at room temperature, and the vial was then charged with 2 mL of 5% ammonium hydroxide and 1 mL of toluene, and vigorously stirred for 20 min. The toluene layer was separated with a Pasteur pipette, dried with anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen. The residue was dissolved in methanol for HPLC analysis.

RESULTS AND DISCUSSION

HPLC separation

A previously published normal-phase partition HPLC method (Sobolev *et al.*, 1995) was very efficient for the separation of peanut stilbenes of different polarities. A modified version of the method was initially used for the study and for the quantitative determination of the metabolites. The method provided baseline separation of the components, and the mobile phase featured excellent dissolving power for analytes of various polarities such as lipids, phenolic acids or glycosides. Furthermore, it helped to avoid any discrimination of the analyte components upon injection into the HPLC system. Recoveries of *trans*-resveratrol (**1**, Fig. 2) were similar to those previously described (Sobolev *et al.*, 1995) and exceeded 95% with spike levels above 100 ng/g. The detection limit for *trans*-resveratrol was 10 ng/g and the PAD detector response at 307 nm was linear up to at least 1.0 μg. The HPLC method made it possible to determine all major components in the peanut extracts and to conclude that the mucigel did not contain any of the known stilbenes that are found in other parts of the peanut plant (Ingham, 1976; Keen and Ingham, 1976; Aguamah *et al.*, 1981; Arora and Strange, 1991; Sobolev *et al.*, 1995; Chen *et al.*, 2002). Typical normal-phase chromatograms of peanut mucigel extract and a fungus-challenged kernel extract are shown in Fig. 3.

However, the original normal-phase HPLC method was not applicable to direct MS^n measurements owing to the high flammability of the mobile phase. Instead, a gradient reversed-phase HPLC was used (Fig. 4). The acidic pH for both normal and reversed phases was chosen to keep the stilbenes in their neutral form (Stojanović *et al.*, 2001), which would provide predictable elution order and better separation with improved peak shape.

Structure characterisation

The HPLC-PAD-MS^n combination was uniquely valuable in this research for structural characterisation of the mucilage compounds owing to the microscale quantities of samples available (Fig. 1). The compounds are labile and light-sensitive, and the quantities obtained were insufficient for NMR analysis.

Combined MS^n data for the reference compounds *t*-arachidin-1 (**3**), *t*-arachidin-2 (**4**), *t*-arachidin-3 (**5**), *t*-IPD (**6**) and the four peaks detected in the mucilage extract are compiled in Tables 1 and 2, respectively. Figure 4 shows the total ion current (TIC) chromatograms of the mucilage extract and of the reference compound mixture isolated from *A. flavus*-challenged peanut kernels. Structures of the reference compounds

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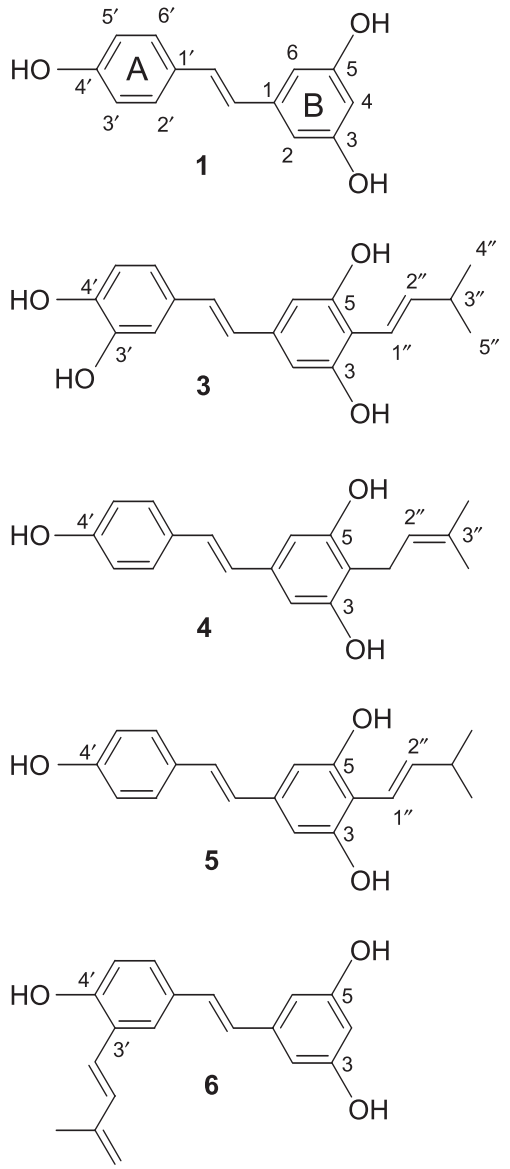

Figure 2 Structures of the reference compounds: **1**, *t*-resveratrol; **3**, *t*-4-(3-methyl-but-1-enyl)-3,5,3′,4′-tetrahydroxystilbene (*t*-arachidin-1); **4**, *t*-4-(3-methyl-but-2-enyl)-3,5,4′-trihydroxystilbene (*t*-arachidin-2); **5**, *t*-4-(3-methyl-but-1-enyl)-3,5,4′-trihydroxystilbene (*t*-arachidin-3); and **6**, *t*-3′-isopentadienyl-3,5,4′-trihydroxystilbene (*t*-IPD).

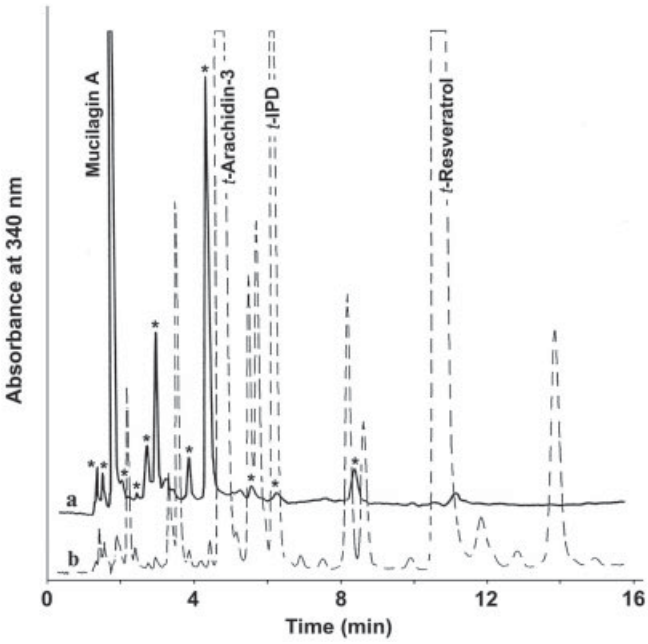

Figure 3 Normal-phase HPLC of peanut root mucilage extract (trace a) and *A. flavus*-challenged peanut kernel extract (trace b). Peaks marked with asterisks are those of stilbenes in the mucilage. (For chromatographic protocol see the Experimental section.)

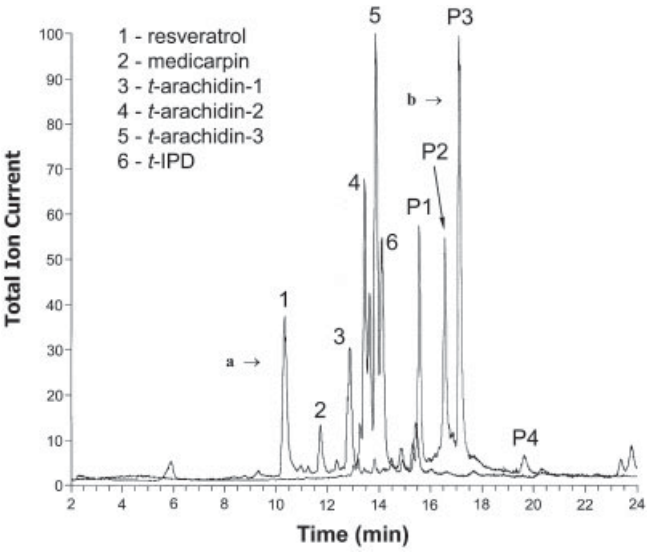

Figure 4 Reverse-phase HPLC-APCI-MS chromatograms of reference compound mixture isolated from *A. flavus*-challenged peanut kernels. (trace a: black) showing peaks of **1**, resveratrol; **2**, medicarpin; **3**, *t*-arachidin-1; **4**, *t*-arachidin-2; **5**, *t*-arachidin-3; **6**, *t*-IPD, and of peanut root mucilage extract (trace b: grey) showing the four principal peaks P1–P4 investigated.

are presented in Fig. 2. As indicated in Fig. 4, four principal peaks (P1–P4) were detected in the mucilage extract. UV spectra taken at the apex scan of each peak are compared with the spectrum of *t*-arachidin-3 in Fig. 5. All spectra uniformly exhibited absorbance maxima in the 335–345 nm range leading to the conclusion that the compounds eluting in these peaks and *t*-arachidin-3 were structurally similar, presenting, in particular, a 4-(3-methyl-but-1-enyl)-group in common. Throughout the manuscript this group is referred to as 'isopentenyl'. UV spectra of *t*-stilbenes such as arachidin-2 (Keen and Ingham, 1976; Aguamah *et al.*, 1981), artoindonesianin N (Hakim *et al.*, 2002) and chiricanine C (Ioset *et al.*, 2001), which possess the 4-(3-methyl-but-2-enyl)-group, are similar to that of *t*-resveratrol (Hillis and Ishikura, 1968; Ingham, 1976) and exhibit two characteristic maxima of

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Table 1 Molecular weights and APCI-MS^n data for the stilbene standards **1** and **3–7** and the products of methylation of **5** showing relative abundance of most abundant ions (m/z^+)

Compound	MW	Scan mode	m/z^+ (relative abundance expressed as percentage of base peak)												
Reference compounds obtained from A. flavus-challenged peanut kernels															
t-Resveratrol (**1**)	228	MS[a]	231 (2)	230 (15)	229 (100)	228 (2)	227 (6)	213 (2)	211 (3)	203 (3)	187 (3)	107 (2)			
		MS^{2b} (229)	229 (41)	211 (38)	201 (3)	193 (5)	187 (7)	183 (13)	171 (5)	165 (2)	145 (2)	135 (100)	119 (16)	111 (4)	107 (12)
t-Arachidin-1 (**3**)	312	MS	315 (2)	314 (20)	313 (100)	295 (15)	293 (2)	241 (7)							
		MS^2 (313)	313 (4)	295 (100)	267 (3)	257 (8)	241 (85)	201 (2)							
		MS^{3c} (313→295)	295 (35)	277 (15)	267 (100)	259 (11)	253 (50)	239 (14)	225 (16)	201 (91)	183 (12)	175 (30)	107 (11)		
t-Arachidin-2 (**4**)	296	MS	299 (3)	298 (20)	297 (100)	295 (2)	243 (2)	242 (3)	241 (20)						
		MS^2 (297)	297 (7)	241 (100)											
		MS^3 (297→241)	241 (100)	195 (86)	223 (69)	199 (56)	213 (46)	167 (14)	197 (12)	169 (11)	157 (9)	185 (8)	171 (8)	147 (6)	181 (6)	183 (5)
t-Arachidin-3 (**5**)	296	MS	299 (2)	298 (20)	297 (100)	295 (4)	293 (2)	242 (2)	241 (12)						
		MS^2 (297)	297 (7)	241 (100)											
		MS^3 (297→241)	241 (88)	226 (5)	223 (87)	213 (60)	205 (6)	199 (47)	197 (9)	195 (100)	185 (10)	181 (6)	171 (6)	169 (14)	167 (14)	157 (7)
t-IPD (**6**)	294	MS	296 (21)	295 (100)	294 (2)	293 (4)	291 (4)	289 (3)	277 (4)	249 (2)	241 (3)				
		MS^2 (295)	295 (100)	277 (55)	267 (17)	262 (8)	259 (5)	253 (15)	239 (29)	237 (14)	235 (5)	225 (4)	221 (5)	211 (13)	185 (28)	173 (11)
		MS^3 (295→277)	277 (84)	275 (4)	262 (100)	259 (68)	249 (67)	248 (15)	247 (6)	235 (9)	234 (5)	231 (16)	223 (5)	221 (16)	207 (16)	193 (8)

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Table 1 (*Continued*)

Compound	MW	Scan mode	m/z^+ (relative abundance expressed as percentage of base peak)													
*Products of methylation of t-arachdin-3 (**5**)*																
Methyl-**5**	310	MS	313	312	311	310	309	257	255							
			(2)	(20)	(100)	(2)	(10)	(2)	(9)							
		MS^2 (311)	255	203												
			(100)	(3)												
		MS^3	255	237	227	223	213	212	211	209	198	195	194	171	169	121
		(311→255)	(60)	(94)	(51)	(66)	(100)	(22)	(17)	(76)	(19)	(17)	(25)	(17)	(18)	(14)
Dimethyl-**5**	324	MS	327	326	325	324	323	271	270	269						
			(3)	(21)	(100)	(5)	(17)	(4)	(5)	(28)						
		MS^2 (325)	325	324	310	307	297	296	293	283	279	269	255	203		
			(13)	(4)	(5)	(4)	(5)	(2)	(3)	(9)	(3)	(57)	(100)	(3)		
		MS^3	269	254	251	241	239	237	227	223	212	209	133	121		
		(325→269)	(17)	(25)	(100)	(43)	(28)	(37)	(32)	(18)	(17)	(45)	(18)	(13)		
Trimethyl-**5**	338	MS	340	339	337	284	283	238	223	210	209	208	195			
			(18)	(85)	(4)	(18)	(100)	(8)	(4)	(3)	(3)	(5)	(5)			
		MS^2 (339)	283	231	189	121										
			(100)	(2)	(6)	(4)										
		MS^3	283	268	255	253	252	251	238	225	223	221	208	175	145	121
		(339→283)	(100)	(37)	(37)	(11)	(20)	(42)	(33)	(15)	(29)	(28)	(14)	(18)	(41)	(46)

[a] MS = full scan.
[b] MS^2 = CID fragmentation of ion shown.
[c] MS^3 = CID fragmentation of sequence of ions shown.

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Table 2 APCI-MS^n data for the novel prenylated stilbenes detected in peaks P1–P4 of the mucilage extract and comparison of MS^3 (339→283) spectra of trimethyl-*t*-arachidin-3 and the principal product detected after methylation of mucilage extract showing relative abundance of most abundant ions (m/z^+)

Compound	%TIC	Scan mode	m/z^+ (relative abundance expressed as percentage of base peak)													
Constituents detected in HPLC-APCI-MS^n analysis of mucilage extract																
Peak **1**	21	MS[a]	366	365	364	363	362	361	359	341	309	307	295	150	141	
			(22)	(67)	(24)	(100)	(4)	(7)	(2)	(2)	(6)	(10)	(2)	(2)	(6)	
		MS^2[b]	307	289	265	203	185	147								
		(363)	(100)	(6)	(2)	(2)	(6)	(2)								
		MS^2	309	308	307	297	296	291	259	253	241	235	203	191		
		(365)	(100)	(22)	(3)	(10)	(2)	(3)	(2)	(30)	(10)	(5)	(2)	(2)		
		MS^3[c]	307	292	289	279	265	264	263	261	251	247	239	237	223	183
		(363→307)	(24)	(25)	(100)	(46)	(87)	(11)	(10)	(27)	(33)	(15)	(8)	(11)	(15)	(13)
Peak **2**	22	MS	380	379	378	377	375	364	363	361	322	321	309	307	141	
			(11)	(42)	(22)	(67)	(10)	(23)	(100)	(13)	(6)	(14)	(6)	(16)	(6)	
		MS^2	377	359	349	335	334	321	303	279						
		(377)	(54)	(7)	(4)	(5)	(2)	(100)	(11)	(2)						
		MS^2	337	323	322	311	305	281	215	201	159	149				
		(379)	(6)	(100)	(4)	(4)	(20)	(3)	(10)	(5)	(3)	(4)				
		MS^3	321	306	303	293	285	279	278	277	275	267	265	251	238	237
		(377→321)	(100)	(13)	(69)	(44)	(10)	(41)	(5)	(33)	(34)	(20)	(63)	(9)	(7)	(8)
Peak **3**	43	MS	327	326	325	324	323	271	270	269	224	209	196	195	194	181
			(3)	(21)	(100)	(2)	(2)	(2)	(12)	(62)	(5)	(2)	(2)	(3)	(4)	(5)
		MS^2	293	282	269	231	219	189	107							
		(325)	(2)	(2)	(100)	(3)	(4)	(8)	(2)							
		MS^3	269	254	241	236	227	224	209	195	188	175	158	131	107	
		(325→269)	(100)	(13)	(32)	(10)	(16)	(17)	(22)	(10)	(32)	(16)	(18)	(18)	(18)	
Peak **4**	3.0	MS	310	309	308	258	254	253	208	179	178	165	160	150	142	
			(14)	(73)	(4)	(4)	(17)	(100)	(9)	(6)	(7)	(6)	(16)	(11)	(5)	
		MS^2	309	303	291	288	280	277	267	266	253	231	218	217	189	179
		(309)	(3)	(2)	(6)	(2)	(2)	(2)	(3)	(2)	(100)	(3)	(4)	(5)	(8)	(3)
		MS^3	253	238	225	222	221	211	210	208	195	193	175	115	91	
		(309→253)	(100)	(85)	(94)	(26)	(42)	(36)	(23)	(39)	(28)	(69)	(23)	(40)	(12)	
Comparison of MS^3 spectra of trimethyl-t-arachidin-3 and principal product detected after methylation of mucilage extract																
Trimethyl-		MS^3	283	268	255	253	252	251	238	225	223	221	208	175	145	121
t-arachidin-3		(339→283)	(100)	(37)	(37)	(11)	(20)	(42)	(33)	(15)	(29)	(28)	(14)	(18)	(41)	(46)
Principal mucilage		MS^3	283	268	255	253	252	251	238	225	223	221	208	175	145	121
methylation product		(339→283)	(100)	(52)	(68)	(31)	(43)	(56)	(47)	(27)	(41)	(33)	(16)	(16)	(64)	(68)

[a] MS = full scan.
[b] MS^2 = CID fragmentation of ion shown.
[c] MS^3 = CID fragmentation of sequence of ions shown.

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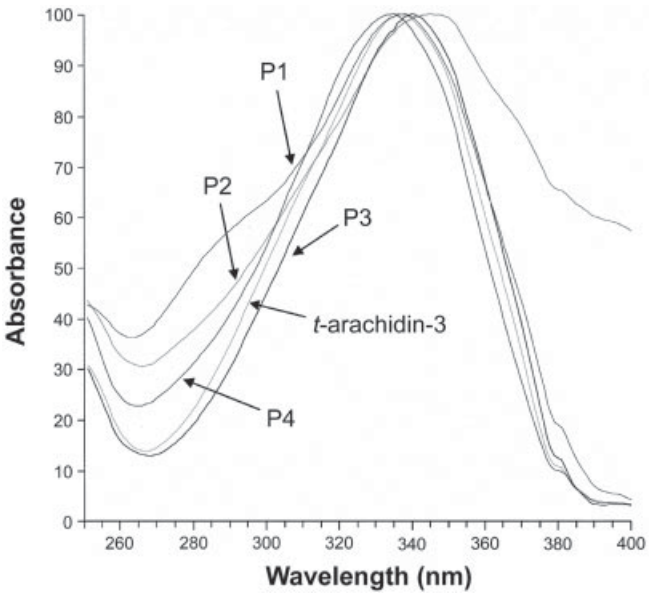

Figure 5 UV spectra of the constituents of the mucilage extract (P1–P4) and of *t*-arachidin-3 isolated from *A. flavus*-challenged peanut kernels.

approximately equal absorbance levels in the 304–324 nm range. It was concluded that all mucilage peak spectra were indicative of stilbenoids in the *t*-configuration. UV spectra of corresponding *cis*-isomers feature broader absorbance maxima with corresponding hypsochromic shifts (10–25 nm; Aguamah *et al.*, 1981; Sobolev *et al.*, 1995). Virtually all natural stilbenes are found in the *t*-configuration (Hillis and Ishikura, 1968; Ingham, 1976; Keen and Ingham, 1976; Aguamah *et al.*, 1981; Cooksey *et al.*, 1988; Sobolev *et al.*, 1995; Ioset *et al.*, 2001; Stojanović *et al.*, 2001; Hakim *et al.*, 2002). These observations, combined with interpretations of MS^n data, lead to the proposed structural assignments of the seven compounds shown in Fig. 6. Two compounds co-eluted in peak P1 and three in peak P2, whilst the largest peak, P3, representing 44% of the TIC, was associated with a single compound: this was also the case with peak P4.

The molecular weights (*M*) were inferred from the $[M+H]^+$ ions detected in MS analyses. $[M+H]^+$ ions were base peaks in MS spectra of all reference compounds (Table 1) and, presumably, of the unknowns. In the

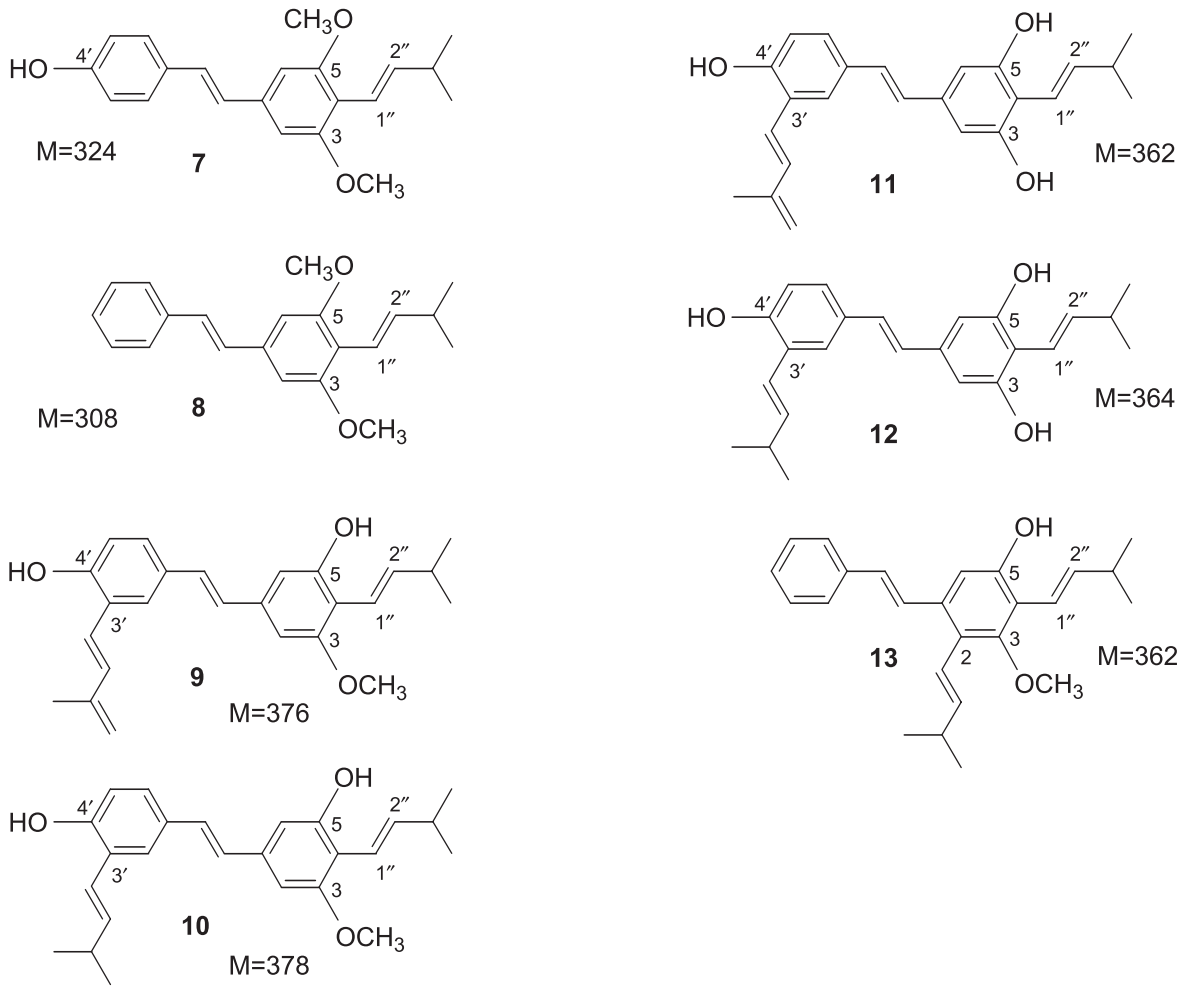

Figure 6 Proposed structures of compounds **7–13** detected in the mucilage extract.

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case of mucilage extract peaks P1 and P2, logical loss arguments lead to the conclusion that there were multiple $[M+H]^+$ ions due to co-elution of structurally similar compounds. In peak P1, $[M+H]^+$ ions at m/z^+ values 363 and 365 were detected, in peak P2 $[M+H]^+$ ions were at 379, 377 and 363, whilst in peaks P3 and P4 they were at 325 and 309, respectively.

Location of an isopentenyl group between two hydroxy or methoxy groups in *ortho* positions on the B stilbene ring and the occurrence of a single hydroxy substituent on the A ring was indicated by MS^2 spectra produced by CID of each $[M+H]^+$. The exception was the compound associated with peak P4 whose spectrum indicated that the A ring was not substituted. The basis for these conclusions was the observation that all MS^2 spectra yielded $[M-55]^+$ as the base peak. The diagnostic neutral loss of 55, corresponding to C_4H_7, was presumably generated by cleavage of the isopentenyl chain at the carbon–carbon bond β to the aromatic ring. This is the characteristic fragmentation pattern for alkyl substituted benzenes by electron impact (EI; Budzikiewicz *et al.*, 1967) and also, apparently, by CID. The $[M-55]^+$ fragment ion has been reported to be diagnostic of an isopentenyl group ortho to two hydroxy groups in EI-spectra of prenylated stilbenes (Keen and Ingham, 1976).

The MS^2 data for the methylation products of *t*-arachdin-3, compiled in Table 1, also indicate that a prominent neutral loss of 55 is observed when one or more of the hydroxy groups is methylated. In trimethyl-*t*-arachidin-3, the base peak was $[M-55]^+$, whilst the relative abundance of this ion was 57% of the base peak in the dimethyl form of the compound.

It appears that $[M-55]^+$ ions are much less prominent in prenyl resveratrol analogues when the isopentenyl group is located at other positions. For example, Su *et al.* (2002) reported that $[M-55]^+$ showed a relative abundance to the base peak of only 21% in the EI spectrum of 3-(γ,γ-dimethylallyl)resveratrol and <10% in that of 5-(γ,γ-dimethylallyl)oxyresveratrol.

Another factor that apparently contributes to the low relative abundance of $[M-55]^+$ is the presence of a pair of *o*-substituted hydroxy groups at the 3′ and 4′ positions of the A stilbene ring. This trend was observed by Su *et al.* (2002) and in the MS^2 spectrum of *t*-arachidin-1 presented here (Table 1). In this spectrum, the $[M-55]^+$ ion exhibited a relative abundance of only 8%, whereas it was the base peak in the spectra of the arachidins 2 and 3 (Table 1). As for the compounds described by Su *et al.* (2002), arachidin-1 is also a piceatannol (oxyresveratrol) analogue. The presence of the second hydroxy group on the A-ring of these compounds apparently promotes the facile loss of water in EI and CID and alternate fragmentation pathways in daughter products.

The number and positions of methoxy groups (Fig. 6), particularly for the compounds associated with mucilage extract peaks P3 and P4, were determined by comparing MS^n spectra of methylation products of *t*-arachidin-3 (**5**) and the mucilage extract. The structure indicated for the compound associated with the principal peak P3 in the mucilage extract mirrored *t*-arachidin-3 when two of its hydroxy groups were methylated. In an attempt to produce an analytical standard for confirmation, *t*-arachdin-3, separated from the reference compound mixture, was partially methylated. The reaction yielded mono-, di- and tri- (fully) methylated *t*-arachidin-3, and the MS^n data for each of these compound are compiled in Table 1.

Upon initial inspection, the dimethyl-derivative obtained appeared to match the unknown compound of peak P3. The reference compound and the unknown had nearly matching retention times and the base peak was at m/z^+ 325 in the MS data acquisition mode. This ion is equivalent to $[M+H]^+$ of the methylated derivative and presumably the unknown. However, there were notable differences in the MS^2 and MS^3 spectra. The ion at m/z^+ 255 was the base peak in the MS^2 spectrum of the dimethyl derivative of *t*-arachidin-3, but this ion was not detected in the MS^2 spectrum of the unknown whose base peak was at m/z^+ 269: the relative abundance of this ion in the dimethyl derivative spectrum was 57%. Loss of an additional 14 mass units to produce an ion at m/z^+ 255 suggested simultaneous demethylation of one of the hydroxy groups and cleavage of C_4H_7 during CID of $[M+H]^+$. A likely explanation of why this was not observed in MS^2 analysis of the unknown was that its methylation pattern differed from that of the *t*-arachidin-3 derivative. UV spectrum of dimethyl *t*-arachidin-3 (λ_{max} 218, 240 sh, 335 nm) did not precisely match that of the unknown (λ_{max} 211, 240, 338 nm); this observation indicates the differences in structures of the two molecules. There are two possible dimethyl derivatives of *t*-arachdin-3. One derivative includes the molecule in which both hydroxy groups on the B ring are methylated. The alternative requires methylation of the A ring hydroxy group in the 4′ position and only one of the B ring hydroxy groups located in either the 3 or the 5 position. Our data suggest that the latter structure was produced by partial methylation of *t*-arachidin-3. This structure was indicated by detection of an m/z^+ at 121 in the MS^3 spectrum of the compound. This ion corresponds to C_8H_9O and was presumably produced by cleavage of the double bond between the aromatic rings. Detection of an ion at m/z^+ 121 was also noted in MS^3 spectra of the mono- and tri-methyl derivatives of *t*-arachidin-3 (Table 1).

It follows that hydroxy groups at the 3 and the 5 positions on the B ring in the unknown were methylated and that there was a lone hydroxy (unmethylated) at the 4′ position on the A ring. Supporting evidence was the detection of an ion of m/z^+ 107 in the MS^3

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spectrum of the compound. This ion, which corresponds to C_8H_7O, was probably a product of cleavage at the aforementioned double bond between the aromatic rings. This type of cleavage was indicated in the MS^2 spectrum of resveratrol, since an ion at m/z^+ 107 was detected (Table 1).

Further support for the structural assignment was provided by the observation that the major product formed by methylation of the mucilage exhibited MS, MS^2 and MS^3 spectra that closely matched those obtained for trimethyl-*t*-arachidin-3. The MS^3 spectra of the two compounds are compared in Table 2. The UV spectrum of the methylated compound was identical in every respect to that of fully methylated *t*-arachidin-3 (λ_{max} 212, 241, 338 nm for both compounds). Given this, the structure, 4-(3-methyl-but-1-enyl)-3, 5-dimethoxy-4′-hydroxy-*trans*-stilbene (**7**) appears to be a logical assignment. The common name mucilagin A is proposed.

With regard to the product eluting in mucilage extract peak P4, cleavage of the double bond between the aromatic rings also appeared diagnostic. An ion at m/z^+ 91 (corresponding to C_7H_7) was detected in the MS^3 spectrum and was indicative of double bond cleavage and formation of the tropylium ion from an unsubstituted A ring (McLafferty and Tureček, 1993). Thus, a structure with methoxy groups at the 3 and 5 positions of the B ring, 4-(3-methyl-but-1-enyl)-3, 5-dimethoxy-*trans*-stilbene (**8**), was proposed.

Magalhães *et al.* (2001) described the isolation of 4-(3-methyl-but-2-enyl)-3, 5-dimethoxy-4′-hydroxy-*trans*-stilbene from roots of *Deguelia hatcshbachii*, a compound that is closely related to mucilagin A. Another compound closely related to mucilagin A was recently reported in extracts of the bark of *Artocarpus gomezianus* bark (Hakim *et al.*, 2002): this compound, named artoindoesianin N, was assigned the structure of 4-(3-methyl-but-1-enyl)-3-methoxy-5-hydroxy-4′-hydroxy-*trans*-stilbene. A compound similar to that proposed for the component associated with peak P4 (structure **8**) has been reported in wood extracts of two *Derris* spp. (Gorham, 1980): this compound possesses a 4-(3-methyl-but-2-enyl)-group whereas **8** comprises a 4-(3-methyl-but-1-enyl)- group.

The proposed structures (Fig. 6) of the compounds that eluted in mucilage peak P1 were *t*-arachidin-3 analogues with an additional isopentenyl group or an isopentadienyl group and exhibited $[M+H]^+$ ions at 365 and 363 (**11**, **12**). The isopentadienyl group is common to the peanut phytoalexin, *t*-3′-isopentadienyl-3,5,4′-trihydroxystilbene (**6**; Cooksey *et al.*, 1988). The two compounds eluting in peak P2 with $[M+H]^+$ at 377 and 379 differed from these compounds by having a methoxy group at the 3 position on the B-ring (**9**, **10**). These structures were supported by the observation that two compounds with $[M+H]^+$ at 405 and 407 were produced upon methylation of the mucilage extract. Methylation of the three hydroxy groups shown in the structures of the peak P1 compounds would probably yield these ions. They would also be produced by methylation of the two hydroxy groups indicated in structures of the peak P2 compounds.

In the case of the compound eluting in peak P2 that yielded $[M+H]^+$ at 363, the most logical assignment is addition of an isopentenyl group to 4-(3-methyl-but-2-enyl)-3-methoxy-5-hydroxy-*t*-stilbene. This compound eluted later than the substance yielding an $[M+H]^+$ ion at 363 in peak P1. Later elution under reverse-phase HPLC conditions suggested that the compound was less polar indicating that this compound probably possessed fewer hydroxy groups. The structure of chiricanine C isolated form the bark of *Lonchocarpus chiricanus* is equivalent to the proposed structure (**13**) associated with $[M+H]^+$ (Ioset *et al.*, 2001). The difference was in the configuration of the hydrocarbon substituent group, which was reported to be 4-(3-methyl-but-1-enyl)- in chiricanine C. We also note that, in the absence of NMR data, the exact configurations of all mucilage peak P1 and P2 compounds remain uncertain. Compounds **7–13** are labile and light-sensitive, and the quantities obtained were insufficient for NMR analysis at this time.

Concentrations of the stilbenes detected in the mucilage extract were estimated using HPLC-PAD data. Calculations were performed using an extinction coefficient of log 4.343 that was derived for the similar compound, *t*-3′-isopentadienyl-3,5,4′-trihydroxystilbene (Cooksey *et al.*, 1988). The concentration of mucilagin A in the mucilage was 250 μg/g, while the concentrations of the other stilbenes totalled 130 μg/g.

The present investigation revealed the presence of seven prenylated stilbenes in the extract of mucilage isolated from peanut root tips and is, to our knowledge, the first report of detection of secondary metabolites in peanut root mucilage. The compound named mucilagin A, 4-(3-methyl-but-1-enyl)-3, 5-dimethoxy-4′-hydroxy-*trans*-stilbene **7**, was the principal constituent. The structural assignment of mucilagin A was strongly supported by our data, but the structures of the constituents **8–13** are tentative; however, the data are sufficient to conclude that these prenylated stilbenoids have not been previously reported in peanuts. Preliminary research demonstrated that peanut root tip with the mucilage removed did not contain any traces of the new stilbenoids **7–13**, in contrast to root tips of some other plants that may accumulate high concentrations of secondary metabolites (Graham, 1991). This suggests that the new compounds are restricted to the mucilage. All but one of the substances detected had a 4′-OH, which has been shown to be strongly associated with the biological activity of stilbenoids (Aguamah *et al.*, 1981; Schultz *et al.*, 1992, 1997; Stojanović *et al.*, 2001; Matsuoka *et al.*, 2002; Sanoh *et al.*, 2003).

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REFERENCES

Aguamah GA, Langcake P, Leworthy DP, Page JA, Pryce RJ, Strange RN. 1981. Two novel stilbene phytoalexins from *Arachis hypogaea*. *Phytochemistry* **20**: 1381–1383.

Arora MK, Strange RN. 1991. Phytoalexin accumulation in groundnuts in response to wounding. *Plant Sci* **78**: 157–163.

Bais HP. 2002. Root-specific elicitation and antimicrobial activity of rosmarinic acid in hairy root cultures of sweet basil (*Ocimum basilicum* L.). *Plant Physiol Biochem* **40**: 983–995.

Bais HP, Park S-W, Weir TL, Callaway RM, Vivanco JM. 2004. How plants communicate using the underground information superhighway. *Trends Plant Sci* **9**: 26–32**.**

Bowen GD, Rovira AD. 1976. Microbial colonization of plant roots. *A Rev Phytopathol* **14**: 121–144.

Budzikiewicz H, Djerassi C, Williams DH. 1967. *Mass Spectrometry of Organic Compounds*. Holden-Day: San Francisco, CA.

Chen R-S, Wu P-L, Chiou RY-Y. 2002. Peanut roots as a source of resveratrol. *J Agric Food Chem* **50**: 1665–1667.

Cooksey CJ, Garratt PJ, Richards SE, Strange RN. 1988. A dienyl stilbene phytoalexin from *Arachis hypogaea*. *Phytochemistry* **27**: 1015–1016.

Dorner JW, Cole RJ, Sanders TH, Blankenship PD. 1989. Interrelationship of kernel water activity, soil temperature, maturity, and phytoalexin production in preharvest aflatoxin contamination of drought-stressed peanuts. *Mycopathologia* **105**: 117–128.

Edwards C, Strange RN. 1991. Separation and identification of phytoalexins from leaves of groundnut (*Arachis hypogaea*) and development of a method for their determination by reversed-phase high-performance liquid chromatography. *J Chromatogr* **547**: 185–193.

Flores HE, Vivanco JM, Loyola-Vargas VM. 1999. 'Radicle' biochemistry: the biology of root-specific metabolism. *Trends in Plant Sci* **4**: 220–226.

Foster RC. 1982. The fine structure of epidermal cell mucilages of roots. *New Phytol* **91**: 727–740.

Gorham J. 1980. The stilbenoids. In *Progress in Phytochemistry*, Reinhold L, Harborne JB, Swain T (eds). Pergamon Press: Oxford; 203–252.

Graham TL. 1991. Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates. *Plant Physisol* **95**: 594–603.

Greaves MP, Darbyshire JF, 1972. The ultrastructure of the mucilaginous layer on plant roots. *Soil Biol Biochem* **4**: 443–449.

Griffin GJ, Hale MG, Shay FJ. 1976. Nature and quantity of sloughed organic matter produced by roots of axenic peanut plants. *Soil Biol Biochem* **8**: 29–32.

Hakim EH, Ulinnuha UZ, Syah YM, Ghisalberti EL. 2002. Artoindonesianins N and O, new prenylated stilbene and prenylated arylbenzofuran derivatives from *Artocarpus gomezianus*. *Fitoterapia* **73**: 597–603.

Hillis WE, Ishikura N. 1968. The chromatographic and spectral properties of stilbene derivatives. *J Chromatogr* **32**: 323–336.

Ingham JL. 1976. 3,5,4′-Trihydroxystilbene as a phytoalexin from groundnuts (*Arachis hypogaea*). *Phytochemistry* **15**: 1791–1793.

Ioset J-R, Marston A, Gupta MP, Hostettman K. 2001. Five new prenylated stilbenes from the root bark of *Lonchocarpus chiricanus*. *J Nat Prod* **64**: 710–715.

Keen NT, Ingham JL. 1976. New stilbene phytoalexins from American cultivars of *Arachis hypogaea*. *Phytochemistry* **15**: 1794–1795.

Magalhães AF, Tozzi AMGA, Magalhães EG, de Souza Moraes VR. 2001. Prenylated flavonoids from *Deguelia hatachbachii* and their systematic significance in *Deguelia*. *Phytochemistry* **57**: 77–89.

Matsuoka A, Takeshita K, Furuta A, Ozaki M, Fukuhara K, Miyata N. 2002. The 4′-hydroxy group is responsible for the *in vivo* cytogenetic activity of resveratrol. *Mutat Res* **521**: 29–35.

McCully ME. 1999. Roots in soil: unearthing the complexities of roots and their rhizospheres. *A Rev Plant Physiol Plant Mol Biol* **50**: 695–718.

McLafferty FW, Tureček F. 1993. *Interpretation of Mass Spectra*. University Science Books: Sausalito.

Paull RE, Jones RL. 1976. Studies on the secretion of maize root cap slime. *Plant Physiol* **57**: 249–256.

Rougier M, Chaboud A. 1985. Mucilages secreted by roots and their biological function. *Isr J Bot* **34**: 129–146.

Sanoh S, Kitamura S, Sugihara K, Fujimoto N. 2003. Estrogenic activity of stilbene derivatives. *J Health Sci* **49**: 359–367.

Schultz TP, Boldin WD, Fisher TH, Nicholas DD, McMurtrey KD, Pobanz K. 1992. Structure–fungicidal properties of some 3- and 4-hydroxylated stilbenes and bibenzyl analogues. *Phytochemistry* **31**: 3801–3806.

Schultz TP, Nicholas DD, Fisher TH. 1997. Quantitative structure–activity relationships of stilbenes and related derivatives against wood-destroying fungi. Recent Res. Devel. *Agric Food Chem* **1**: 289–299.

Sobolev VS, Cole RJ, Dorner JW, Yagen B. 1995. Isolation, purification, and liquid chromatographic determination of stilbene phytoalexins in peanuts. *J AOAC Int* **78**: 1177–1182.

Stojanović S, Sprinz H, Brede O. 2001. Efficiency and mechanism of the antioxidant action of *trans*-resveratrol and its analogues in the radical liposome oxidation. *Arch Biochem Biophys* **391**: 79–89.

Strange RN, Ingham JL, Cole DL, Cavill ME, Edwards C, Cooksey CJ, Garratt PJ. 1985. Isolation of the phytoalexin medicarpin from leaflets of *Arachis hypogaea* and related species of the Tribe *Aeschynomeneae*. *Z Naturforsch* **40c**: 313–316.

Su B-N, Cuendet M, Hawthorne ME, Kardano LBS, Riswan S, Fong HHS, Mehta RG, Pezzuto JM, Kinghorn AD. 2002. Constituents of the bark and twigs of *Artocarpus dadah* with cyclooxygenase inhibitory activity. *J Nat Prod* **65**: 163–169.

Walker TS, Bais HP, Grotewold E, Vivanco JM. 2003. Root exudation and rhizosphere biology. *Plant Physiol* **132**: 44–51.

Werker E, Kislev M. 1978. Mucilage on the root surface and root hairs of sorghum: heterogeneity in structure, manner of production and site of accumulation. *Ann Bot* **42**: 89–816.

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